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Steroid Derivatives Reduce the Activity of Antithrombin III and the Affinity to Sulfated Polysaccharides

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Summary. **We examined the denaturing effects of deoxycholate and estrogen-3-monosulfate on antithrombin III (AT III) *in vitro*. These compounds reduced in parallel both the immunological and anticoagulant activity of AT III at high concentrations, and such denaturing effects were considered to be due to their detergent-like effect. These compounds made AT III heat-labile and reduced the affinity of AT III to sulfated polysaccharides such as heparin attached to agarose gel at lower concentrations, which showed no direct denaturation yet. These results indicate that such compounds bind to a lipophilic portion, which may be involved with the function of AT III.**

INTRODUCTION

Since steroid derivatives have a cholate-like molecular structure (a large lipophilic portion with few hydrophilic ones), we consider these compounds to bind directly and nonspecifically to proteins in the manner of a detergent, and thus possibly affect their various functions. Nagasawa et al. have reported previously that conjugated estrogens at extremely high concentrations reduced both the immunological and anticoagulant activity of AT III *in vitro*.[1,2]

AT III is a stable and hydrophilic protein. In terms of primary structure, its molecule can be roughly divided into two functional domains;[3,4] the heparin-binding, and the thrombin-binding one (Fig. 1). Five major lipophilic portions are located in the latter, and the former one is relatively hydrophilic in contrast. Furthermore, there is a fragmentary sequence homology between AT III[3] and the estrogen receptor molecule.[5] However, such homological sequences were found in only a few parts of AT III and the other proteins (plasma enzymes and ion channels, data not shown). This may indicate that the two proteins are not far distant from the molecular phylogenic viewpoint.

If such a sequence is closely related to the steroid binding activity, steroid derivatives might affect a certain function of AT III even at a lower concentration rather than simply acting as a detergent. In this study, we investigated the effect of estrogens on AT III and on the affinity of AT III to sulfated polysaccharide from the viewpoint of this detergent activity.

MATERIALS AND METHODS

Chemicals and reagent solutions

HEPES, bovine serum albumin (BSA), bovine thrombin (IIa), S-2238 (H-D-Phe-Pip-Arg-pNA), heparin (porcine intestinal mucosa), heparin monosulfate (HMS, bovine kidney), estrone-3-monosulfate (EMS), cholate-agarose and other sulfated steroid derivatives were purchased from Sigma (St. Louis, USA). Deoxycholate (DOC), phosphotungstate (PTA) and other reagents were products of Wako Pure Chemicals (Osaka, Japan). Heparin-Sepharose CL-6B and sulfated-Cellulofine (sulfated cellulose) were obtained from Pharmacia (Uppsala, Sweden) and Chisso Co. (Kumamoto, Japan), respectively.

IIa (1.2 NIH. U/ml, mixed with heparin (3U/ml) and 0.01% (w/v) BSA), S-2238 (1 mM) and all other reagents were dissolved in Buffer A (150 mM NaCl and 20 mM HEPES, pH 7.2) and kept at 4℃. One hundred μl of a reagent solution (or homogeneous suspension) mixed with 100 μl of AT III solution (2U/ml) was incubated (10 min, 37℃), and then the immunological and anticoagulant activities of AT III in

Table 1. Effects of various compounds on the anticoagulant activity of AT III

Compound	Final concn.	Anticoagulant activity of AT III (%)
None		100.0
Estrone-3-Sulfate	0.5 mM	98.5
	2.5m M	20.1
+ PTA	300 μM	55.7
+ Heparin	2 μM	31.2
Deoxycholate	0.5 mM	98.4
	2.5 mM	60.1
+PTA	300 μM	95.7
+Heparin	2 μM	68.5
Estradiol Sulfate	2.5 mM	88.4
Cholesterol Sulfate	2.5 mM	58.6
Cortisol Sulfate	2.5 mM	85.0
Cholate	2.5 mM	82.1
Triton X-100	2.5%(v/v)	98.2
Tween 20	1.0%(v/v)	95.7
Brij 56	2.5%(v/v)	96.8
Nonidet P-40	2.5%(v/v)	98.1

Various compounds shown in this table were mixed with purified AT III, and the residual anticoagulant activity of AT III was measured. The results are expressed as values relative to the control.

the resulting mixture were measured.

Preparation of AT III and calculation of hydropathic index

We employed McKay's method.[6] Dextran sulfate (0.1% (w/v)) and $CaCl_2$ (50 mM) were added to citrated human plasma. After removing lipoproteins by centrifugation, the plasma was applied to a heparin-Sepharose column. After a linear salt gradient elution (0.15-2 M NaCl), the AT III fraction (purity =97%) was washed with Buffer A by ultrafiltration and then used without further purification (20 U/ml, stored at −20°C). The hydropathic index and sequence homology of AT III and the estrogen receptor molecule were calculated by the method of Kyte and Doolittle,[7] using special software (GENETYX; SDC Software Co., Tokyo, Japan).

Assay of immunological and anticoagulant activities of AT III

The amount of immunoreactive AT III protein was measured by latex-immunoturbidimetry at 950 nm, using a kit (LPIA AT III) available from Dia-iatron (Tokyo, Japan) and a rate-turbidimeter (LPIA-100M). The anticoagulant activity of AT III was measured as the heparin-cofactor activity.[8] A sample adequately diluted with Buffer A (100 μl) and the IIa solution (200 μl) were incubated for 5 min at 37°C. After the S-2238 solution (200 μl) was added, and the resulting mixture was further incubated (37°C, 5 min). Finally, 2 ml of 2% (w/v) citric acid solution was added to

Table 2. Effect estrone-3-sulfate and deoxycholate on AT III activity

	Anticoagulant activity (%) / Immunoreactivity (%)						
Estrone-3-Sulfate	0	2.5	5.0	7.5	10.0	12.5	15.0mM
Purified AT III	100/100	21/27	12/14	<5/10	<5/8	<5/6	<5/<5
+ PTA (0.5mM)	98/101	56/58	29/32	9/17	<5/11	<5/9	<5/<5
AT III in Plasma	100/100	99/99	85/90	61/68	42/48	25/29	< 5 / 9
+ PTA (0.5mM)	102/103	96/98	87/92	67/74	49/57	30/37	9/14
Deoxycholate	0	2.5	5.0	7.5	10.0	12.5	15.0mM
Purified AT III	100/100	61/65	26/26	11/14	<5/10	<5/8	<5/6
+ PTA (0.5mM)	102/97	96/98	76/80	36/42	24/28	15/17	9/14
AT III in Plasma	100/100	99/99	93/97	86/90	71/72	48/54	21/30
+ PTA (0.5mM)	101/100	96/98	92/96	87/93	76/78	52/65	35/39

Experimental procedure was the same as that for data in Table 1, but either citrated plasma or purified protein was used as a source of AT III. Both anticoagulant activity and immunoreactivity were measured. The results are expressed as values relative to the control (underlined).

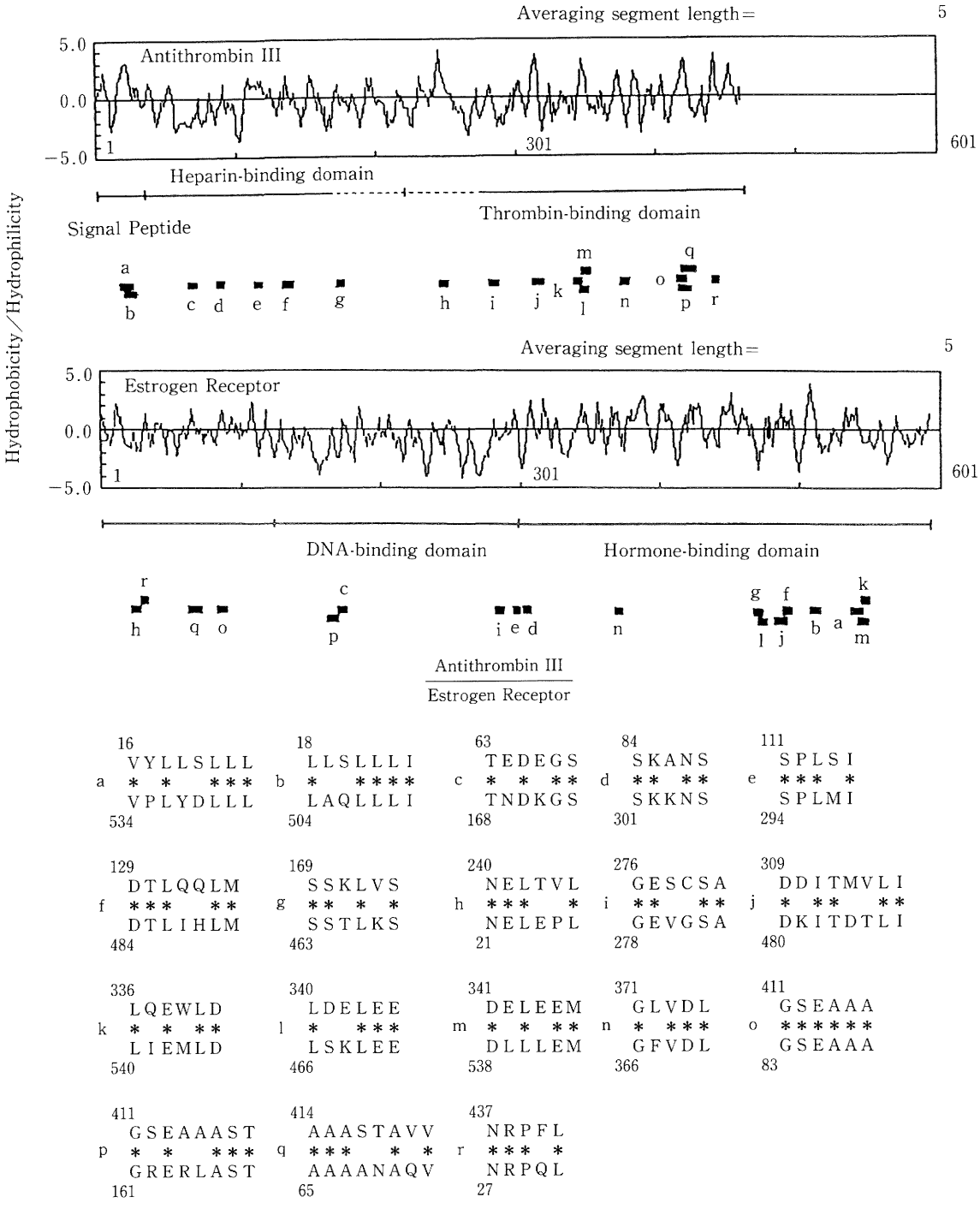

Fig. 1. Hydrophobicity of AT III molecule.
The hydropathic index of AT III and the estrogen receptor molecule is in the upper panel. Sequence homology (> 60%) between the two proteins is shown in the lower panel and indicated by lettering.

stop the reaction. The anticoagulant activity of AT III was estimated from the residual activity of IIa (A_{405}/5 min) . The standard plasma (Organon Teknica Co.; New Jersey, USA) is considered to have an AT III activity of 1U/ml (approximately 300 μg AT III/ml plasma).

Affinity gel adsorption

HMS was attached on Sepharose 4B according to the method of Iverius.[9] HMS-Sepharose, heparin-Sepharose, sulfated-Cellulofine, cholate-agarose and Sepharose 4B (used as the control) prewashed with 2 M NaCl were washed again extensively with Buffer A, and suspended in the same buffer (gel : buffer=1 : 1). Fifty μl of AT III solution (4 U/ml), 50 μl of a reagent solution and 100 μl of any one gel slurry were mixed in this order, and the mixture was incubated (1 h, 37°C) with gentle mixing. After the gel was sedimented by centrifugation (2000xg, 5 min), the amount of unbound AT III was determined by measurement of protein in the supernatant, which was in turn determined by the bicinchoninic acid method[10] (using BSA as a standard).

Assay of plasma enzymes and apolipoproteins

The assay methods to determine the following enzymes are shown in the references: alkaline phosphatase (ALP[11]), amylase (AMY[12]), choline esterase (CHE[13]), creatine phosphokinase (CPK[14]), glutamate-oxaloacetate transaminase (GOT[11]), glutamate-pyruvate transaminase (GPT[11]), γ-glutamyl transpeptidase (GTP[15]), leucine aminopeptidase (LAP[16]) and lactate dehydrogenase (LDH[11]). Quantitative determination of apolipoproteins (A-I, A-II, B, C-II, C-III, E) was done by immunoturbidimetry,[17] using a kit available from Daiichi Pure Chemicals (Tokyo, Japan).

RESULTS

Table 1 shows that the anticoagulant activity of purified AT III was reduced by the addition of sulfated steroid derivatives, but non-soluble compounds such as non-esterized estrone showed no effect (data not shown). Among the compounds, EMS had the most potent effect. However, the denaturing effect of polyoxyethylene detergent such as Triton X-100 on AT III was weak when compared with EMS and DOC, whereas the solubilizing potency of these deter-

Table 3. Effect of estrone-3-sulfate on various plasma proteins

Estrone-3-Sulfate	10mM	+PTA (0.5 mM)	+Heparin (2 μM)	20mM	+PTA (0.5 mM)	+Heparin (2 μM)
			Relative Activity (%)			
Enzyme						
ALP	104.0	101.2	98.3	98.6	95.2	97.1
AMY	82.5	80.8	91.7	82.0	80.3	81.1
CHE	93.1	90.8	91.8	86.4	84.0	85.9
CPK	68.5	62.2	67.3	20.1	18.4	19.0
GOT	82.3	83.7	81.4	58.9	56.5	57.4
GPT	10.5	8.8	8.3	<5.0	<5.0	<5.0
GTP	88.2	89.0	85.6	64.7	65.4	62.8
LAP	76.7	75.1	77.5	33.8	30.9	31.6
LDH	23.6	19.9	23.7	9.9	7.1	8.2
Apolipoprotein						
A-I	121.7	129.2	118.5	140.6	144.8	135.1
A-II	106.3	111.8	105.2	115.2	118.5	109.2
B	65.7	65.1	69.4	26.0	33.2	35.9
C-II	101.2	103.4	98.5	87.7	89.5	88.4
C-III	95.1	98.6	99.2	82.8	80.1	83.7
E	79.4	82.5	82.3	52.1	49.8	50.5

Estrone sulfate, PTA and heparin were mixed with human serum, and the activities of various enzymes and immunoreactivities of apolipoproteins were determined. The results are expressed as values relative to the control.

gents to BSA in ethanol solution was greater than that of the two (data not shown). As shown in Table 2, both the immunological and anticoagulant activity of AT III was concomitantly reduced by the addition of EMS or DOC, but the former was slightly less inhibited than the latter, suggesting the presence of some residual epitopes not closely related to the latter activity.

Addition of PTA or heparin (cofactors of AT III[18,19]) reduced the denaturing effect, especially that of DOC, but such a protective effect of PTA was marked only when the concentration of DOC was low (data not shown). On a molar basis, the protective effect of heparin was stronger than that of PTA, but the effect of PTA was greater on the basis of the cofactor activity. This is perhaps because PTA is a small molecule and hardly affected by steric hindrance.[19,20]

EMS and DOC showed slightly less marked denaturing effects on AT III in plasma, and the protective effect of PTA was also reduced (data not shown). Inactivation of AT III by free fatty acid was also reduced in the presence of BSA.[21] These results indicate that various proteins in plasma bind to these compounds and diminish their effects.

In addition, the effects of estrogens were not specific to AT III. Table 3 shows the effect of EMS on enzymes which are measured in clinical tests. EMS markedly reduced the activities of LDH, CPK and GPT, and moderately reduced those of GOT and LAP. ALP, GTP, CHE and AMY were not inhibited significantly. The immunoreactivities of apolipoproteins A-I and A-II were somehow potentiated by the addition of EMS. C-II and C-III were not markedly influenced, whereas B and E were markedly reduced. However, neither PTA nor heparin showed any protective effect in all the cases tested. DOC showed a similar, but rather weak denaturing effect on these enzymes or proteins (data not shown).

Table 4. Effect of deoxycholate on the thermal stability of AT III

	Anticoagulant activity of AT III (%)					
Temperature (℃)	37	50	55	60	65	70
None	100	103	95	39	7	<1
+ PTA (300 μM)	105	101	90	29	4	<1
+Heparin (2 μM)	102	102	95	37	7	2
Deoxycholate (0.5 mM)	101	72	17	2	<1	<1
+ PTA (300 μM)	105	77	12	3	<1	<1
+ Heparin (2 μM)	103	75	15	4	<1	<1

Purified AT III was mixed with various reagents, and then incubated at the desired temperature. The residual anticoagulant activity of AT III was measured and expressed as values relative to the control (underlined).

Table 5. Effects of steroid derivatives on affinity gel adsorption of AT III

Concentration	0	0.1	0.2	0.5	1.0	2.0 (mM)
	(Estrone-3-Sulfate/Deoxycholate)					
Affinity Ligand (Ligand Contents/ml Gel)	Unbound AT III (%)					
Heparin	<5	<5	9	14	20	28
(750 μg)	<5	11	14	29	48	81
Dextran Sulfate*	32	39	46	57	65	78
(8 μmole SO_4^{2-})	34	51	59	77	84	95
Heparan Sulfate	74	85	92	>95	>95	>95
(2 mg)	75	94	>95	>95	>95	>95
Cholic Acid	>95	>95	>95	94	91	89
(2 μmole)	>95	>95	>95	93	90	86

*Sulfated cellulose (sulfated-Cellulofine) was used.
Purified AT III was mixed with estrone sulfate or deoxycholate, and the affinity-gel slurry was added to this mixture. Finally, the amount of unbound AT III was determined and expressed as a ratio relative to the total amount.

However, EMS or DOC latently affected AT III at lower concentrations. Table 3 indicates that DOC made AT III heat-labile even at a lower concentration which showed no direct denaturing effect at 37℃. In this case, neither PTA nor heparin protected the protein from inactivation. EMS produced similar results at a lower temperature than that for DOC (data not shown).

Finally, we studied the effect of estrogens on the affinity of AT III to sulfated polysaccharide. As shown in Table 5, AT III was well adsorbed on heparin-Sepharose, sulfated-Cellulofine and HMS-Sepharose in that order. This may be because heparin contains a special sugar sequence and HMS contains a lower amount of sulfonic radicals per molecule, both of which are closely related to AT III affinity.[18] The affinity of AT III to such polysaccharides on the gel was reduced by the addition of DOC and EMS, even at a lower concentration which showed no denaturing effect. EMS and DOC seemed to bind to AT III, but cholate-agarose did not bind a significant amount of AT III. However, the affinity of AT III to this gel became slightly higher as the concentration of DOC increased. The effects of other compounds on affinity gel adsorption are summarized in Table 6. Steroid derivatives showed similar effects with DOC.

Table 6. Effects of various compounds on the affinity gel adsorption

Reagent	Final Concn.	Unbound AT III (%)
None		<5.0
NaCl	500 mM	6.0
	1500 mM	>95.0
Heparin	2 μM	24.8
	10 μM	75.9
PTA	300 μM	45.1
	1 mM	>95.0
Estrone Sulfate	0.5 mM	56.6
	1 mM	83.7
Estradiol Sulfate	0.5 mM	<5.0
Cholesterol Sulfate	0.5 mM	12.6
Cortisol Sulfate	0.5 mM	41.9
Deoxycholate	0.5 mM	31.2
Cholate	0.5 mM	<5.0
Triton X-100	2.5%(v/v)	<5.0

Experimental procedure was the same as that for data in Table 5, but sulfated cellulose gel was used throughout because of its relatively low affinity for AT III thus simplifying the comparison of the effects of various reagents.

DISCUSSION

The addition of steroid derivatives to AT III caused a dose-dependent reduction of both the immunological and anticoagulant activity of AT III, in accordance with the results of Nagasawa et al.[1,2] However, the potency of such a denaturing effect appeared to be related to be hydrophile-lipophile balance of the molecule. In addition, the denaturing effects of EMS and DOC were reduced in the presence of plasma proteins, and were not specific to AT III. We thus considered that such denaturation effects caused by a high concentration of steroid derivatives were due to their detergent-like, rather than hormonal, action.

Such compounds seem to bind to AT III molecules even at a lower concentration, as well as making a complex of AT III and fatty acid.[21] In a study of IIa-induced platelet aggregation, Nagasawa et al. found that the anticoagulant activity of AT III was inhibited by a very small amount of estrogen.[22] We found that a low amount of EMS made AT III heat-labile and lowered the affinity of AT III to sulfated polysaccharide. These results indicate that the functional activity of AT III is already reduced by a low concentration of steroid derivatives not completely inactivating AT III. Heparin and PTA protected AT III from the denaturation. Although it is still unclear whether or not steroid derivatives bind more selectively the heparin-binding domain, the bindings of heparin and DOC to AT III may exert an influence to each other.

Contrary to the solubilizing potency, the denaturing effect of the polyoxyethylene detergents on AT III was weak. This is perhaps because the detergent-binding area (possibly the lipophilic portion on the surface) of this molecule is too small to allow the long chain-like molecule of detergent to interact with effectively. Judging from its primary structure (Fig. 1), we suppose that such a lipophilic area is interspersed among hydrophilic surface structure like a crevice, where steroid derivatives are able to bind like a wedge. Furthermore, the affinity of AT III to cholate-agarose gel became slightly higher as the concentration of DOC increased. This may suggest that the conformation of the AT III molecule is already altered by binding undenaturative amount of DOC, and then another lipophilic portion hidden inside the molecule comes to be exposed to surface. We thus consider that affinity chromatography is a useful "tool" not only for the purification of various materials, but also for a tentative analysis of conformation change of several proteins.

When sulfated polysaccharides such as heparin bind with AT III, its molecular conformation is altered, resulting in the activation of this protein.[18] It is generally believed that heparin-like polysaccharides such as HMS coating vascular endothelial surface deeply concern the physiological local blood anticoagulation via activating AT III.[23,24] When compared with heparin, the acidity of the HMS molecule is lower, and its affinity to AT III is very much weaker than that of heparin. This suggests that the binding of AT III to HMS is easily broken by minor conditional changes, such as hyperlipemia and administration of excess amounts of steroid derivatives, which may lead to hypercoagulability *in vivo*.

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